JC03 Rec'd PCT/PTO 25 MAY 2001

FORM PTO-1390 (REV 10-96) — U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER: 01307/HG

U.S. APPLICATION NO. (If known, see 37 CFR 1.5): 09/856790

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/JP99/06847	7 December 1999	7 Dec. '98; 26 March '99; 27 May '99

TITLE OF INVENTION: METHOD FOR QUANTITATIVELY DETERMINING HYDROGEN SULFIDE OR SULFIDE IONS, AND METHOD FOR QUANTITATIVELY DETERMINING A SPECIFIC SUBSTANCE UTILIZING IT

APPLICANT(S) FOR DO/EO/US: Hiroyuki EBINUMA; Koji USHIZAWA

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. [X] This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. [ ] This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. [ ] This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. [X] A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. [X] A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   - a. [ ] is transmitted herewith (required only if not transmitted by the International Bureau).
   - b. [XX] has been transmitted by the International Bureau.
   - c. [ ] is not required, as the application was filed in the United States Receiving Office (RO/US).
6. [X] A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. [ ] Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
   - a. [ ] are transmitted herewith (required only if not transmitted by the International Bureau).
   - b. [ ] have been transmitted by the International Bureau.
   - c. [ ] have not been made; however, the time limit for making such amendments has NOT expired.
   - d. [ ] have not been made and will not be made.
8. [ ] A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. [X] An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. [ ] A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. below concern document(s) or information included:

11. [X] An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. [X] An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. [X] A FIRST preliminary amendment.
    [ ] A SECOND or SUBSEQUENT preliminary amendment.
14. [ ] A substitute specification.
15. [ ] A change of power of attorney and/or address letter.
16. [X] Other items or information: Copy of:
    - (i) Drawings, 2 sheets, for the Examiner's convenience
    - (ii) NOTICE PCT/IB/308
    - (iii) WO 00/34771, the 18-month publication of PCT/JP99/06847
    - (iv) Int. Prel. Exam. Report PCT/IPEA/409

Express Mail Mailing Label No.: EL 797 383 225 US

Date of Deposit: May 25, 2001

I hereby certify that this paper and any papers identified herein is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10 on the date indicated above and is addressed to the Assistant Commissioner for Patents, Washington, D.C. 20231

Lori Valdes

Lori Valdes

U.S. APPLICATION NO. (if known, see 37 CFR 1.5) 09/856790 | INTERNATIONAL APPLICATION NO. PCT/JP99/06847 | ATTORNEY'S DOCKET NUMBER 01307/HG

JC18 Rec'd PCT/PTO 25 MAY 2001

17. [X] The following fees are submitted:

	CALCULATIONS	PTO USE ONLY
BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)):		
Search Report has been prepared by the EPO or JPO $860.00		
International preliminary examination fee paid to USPTO (37 CFR 1.482) $690.00		
No international preliminary examination fee paid to USPTO (37 CFR 1.482) but international search fee paid to USPTO (37 CFR 1.445(a)(2)) $750.00		
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO $1.000.00		
International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2)-(4) $100.00		
ENTER APPROPRIATE BASIC FEE AMOUNT =	$ 860.00	
Surcharge of **$130.00** for furnishing the oath or declaration later than [ ] 20 [ ] 30 months from the earliest claimed priority date (37 CFR 1.492(e)).	$	

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	14 - 20 =	0	x 18.00	$ —	
Independent claims	2 - 3 =	0	x 80.00	$ —	
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ 270.00	$ —	
TOTAL OF ABOVE CALCULATIONS =				$ 860.00	
Reduction of 1/2 for filing by small entity, if applicable. Verified Small Entity Statement must also by filed (Note 37 CFR 1.9, 1.27, 1.28).				$	
SUBTOTAL =				$ 860.00	
Processing fee of **$130.00** for furnishing the English translation later than [ ] 20 [ ] 30 months from the earliest claimed priority date (37 CFR 1.492(f)). +				$	
TOTAL NATIONAL FEE =				$ 860.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$40.00** per property +				$ 40.00	
TOTAL FEES ENCLOSED =				$ 900.00	
				Amount to be: refunded	$
				charged	$

a. [X] A check in the amount of $ 900.00 to cover the above fees is enclosed.

b. [ ] Please charge my Deposit Account No. ______ in the amount of $ ______ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. [X] The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 06-1378. A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

FRISHAUF, HOLTZ, GOODMAN, LANGER & CHICK, P.C.
767 Third Avenue - 25th Floor
New York, NY 10017-2023
Tel. No. (212) 319-4900
Fax No. (212) 319-5101

Date: May 25, 2001

01933
PATENT TRADEMARK OFFICE

SIGNATURE

Herbert Goodman
NAME

17,081
REGISTRATION NUMBER

09/856790

JC18 Rec'd PCT/PTO 25 MAY 2001

Attorney Docket No. 01307/HG

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant(s): Hiroyuki EBINUMA et al

Serial No. : U.S. National Phase Appln. based on PCT/JP99/06847

Filed : Concomitantly herewith

For : METHOD FOR DETERMINATION OF HYDROGEN SULFIDE OR SULFIDE ION AND METHOD....

Express Mail Mailing Label No.: 797 383 225 US
Date of Deposit: May 25, 2001

I hereby certify that this paper is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10 on the date indicated above and is addressed to the Assistant Commissioner for Patents, Washington, D.C. 20231

Lori Valdes

Lori Valdes

In the event that this Paper is late filed, and the necessary petition for extension of time is not filed concurrently herewith, please consider this as a Petition for the requisite extension of time, and to the extent not tendered by check attached hereto, authorization to charge the extension fee, or any other fee required in connection with this Paper to Account No. 06-1378.

PRELIMINARY AMENDMENT FILED CONCOMITANT WITH APPLICATION

Assistant Commissioner for Patents
Washington, D.C. 20231

S I R :

Please amend the application as follows:

IN THE SPECIFICATION:

Page 1, at the top of the page, above "Technical Field" and below the title, insert:

--This application is a national phase application of International Application No. PCT/JP99/06847 (not published in English) filed December 7, 1999.--

Respectfully submitted,

Frishauf, Holtz, Goodman,
Langer & Chick, P.C.
767 Third Ave., 25th floor
New York, NY 10017-2023
Telephone: (212) 319-4900
Facsimile: (212) 319-5101
HG/lpv

HERBERT GOODMAN
Reg. No. 17,081

2/PRTS

09/856790

JC18 Rec'd PCT/PTO 2 5 MAY 2001

SPECIFICATION

Method for quantitatively determining hydrogen sulfide or sulfide ions, and method for quantitatively determining a specific substance utilizing it

Technical Field

The present invention relates to a method for quantitatively determining hydrogen sulfide or sulfide ions in a sample conveniently with high sensitivity, by utilizing an acceleration or inhibition reaction by hydrogen sulfide or sulfide ions against formation of complexes between metal ions and a metal indicator; and a method for quantitatively determining a specific substance in a sample conveniently with high sensitivity, wherein hydrogen sulfide is formed from the specific substance and the hydrogen sulfide or sulfide ions derived therefrom are measured by utilizing the above quantitative determination method.

Background Art

Sulfur is one of elements which take important roles in nature. Particularly, it takes a substantial role as a constituting element for cysteine and methionine as sulfur-containing amino acids. Further, it has been known that a large sulfur cycle is carried out between plants and

animals. In the plants, sulfur is taken in the form of sulfate ions and reduced to sulfide ions, and then subjected to cysteine synthesis and further methionine synthesis. In the animals, methionine is ingested from foods by food cycle and metabolized to cysteine in vivo. In this metabolization stage, homocysteine is formed as an intermediate.

Homocysteine is an intermediary metabolite which is scarcely present at the normal condition. However, it has been reported that when its concentration in blood is at a high level, the rate of occurrence of coronary disease and cerebral apoplexy is high. Accordingly, the homocysteine amount in blood has been recognized to be a risk factor useful for predicting the occurrence of thromboembolism such as myocardinal infarction or cerebral infarction, or arteriosclerosis.

Further, since cysteine is an amino acid formed by metabolism of methionine, it may be an auxiliary index for grasping the causes of metabolic error of homocysteine.

Furthermore, an enzyme is known which has a function of acting on homocysteine or cysteine and conducting decomposition or substitution, thereby forming hydrogen sulfide. However, even if it is attempted to quantitatively determine homocysteine or cysteine by utilizing such an enzyme, since there is no method for measuring the formed hydrogen sulfide conveniently with high sensitivity, no practical

method has been found in which this enzyme is used for quantitatively determining homocysteine or cysteine.

On the other hand, hydrogen sulfide or sulfide ions derived therefrom are important as an index of environmental pollution such as air pollution or water pollution of rivers. The state of pollution can be confirmed by measuring the hydrogen sulfide or sulfide ions derived therefrom in samples.

As conventional methods for measuring hydrogen sulfide or sulfide ions derived therefrom, the following methods are known. For example, as a method of utilizing a color developer, a method wherein 2,2'-dipyridyldisulfide (Svenson, Anal. Biochem., 107; 51-55(1980)) or sodium nitroprusside is used, a method wherein N,N-dimethyl-P-phenylenediamine and ferric chloride are reacted in a strong acidic condition to form methylene blue and blue color development is detected (methylene blue method), a method wherein fading amount and rate of a dye (toluidine blue or methylene blue) is measured by using cerenium as a catalyst (Mousavi et al, Bull. Chem. Soc. Jpn, 65;2770-2772(1992), Gokmen et al, Analyst, 119;703-708 (1994)), and the like are known. However, these methods are hardly adequate from the viewpoints of convenience and sensitivity.

Disclosure of Invention

Accordingly, it is an object of the present invention

to provide a more convenient and highly sensitive method for quantitatively determining hydrogen sulfide or sulfide ions. It is another object of the present invention to provide a method for quantitatively determining a specific substance in a sample conveniently with high sensitivity, wherein hydrogen sulfide is formed from the specific substance and the hydrogen sulfide or sulfide ions derived therefrom are measured by utilizing the above quantitative determination method.

The present inventors have conducted intensive studies to accomplish the above objects, and found that hydrogen sulfide or sulfide ions in a sample can be quantitatively determined conveniently with high sensitivity by utilizing an inhibition or acceleration action by the hydrogen sulfide or sulfide ions against color development between metal ions and a metal indicator.

Namely, the present invention provides a method for quantitatively determining hydrogen sulfide or sulfide ions, which comprises adding to a sample containing hydrogen sulfide or sulfide ions, metal ions or a compound which liberates said metal ions and a metal indicator which reacts with the metal ions and resultingly undergoes color development, wherein the color development is accelerated or inhibited by the hydrogen sulfide or sulfide ions; and measuring the degree of color development of the metal indicator.

The present invention further provides a method for

quantitatively determining a specific substance, which comprises adding to a sample containing a specific substance, a component which acts on the specific substance so that the specific substance forms hydrogen sulfide or sulfide ions, metal ions or a compound which liberates said metal ions, and a metal indicator which reacts with the metal ions and resultingly undergoes color development, wherein the color development is accelerated or inhibited by the hydrogen sulfide or sulfide ions; and measuring the degree of color development of the metal indicator.

According to the present invention, it is possible to quantitatively determine hydrogen sulfide or sulfide ions conveniently with high sensitivity, by utilizing an acceleration or inhibition reaction by the sulfide ions against formation of complexes between metal ions and a metal indicator. It is further possible to quantitatively determine a specific substance in a sample conveniently with high sensitivity, by adding a component which acts on the specific substance so that the specific substance forms hydrogen sulfide or sulfide ions to the sample, and measuring the formed hydrogen sulfide utilizing the above reaction.

Brief Description of Drawings

Fig.1 is a graph showing the results of quantitative determination of sulfide ions by a color development-

inhibiting method.

Fig.2 is a graph showing the results of quantitative determination of sulfide ions by a color development-accelerating method.

Fig.3 is a graph showing the results of quantitative determination of homocysteine by a color development-accelerating method.

Fig.4 is a graph showing the results of quantitative determination of cysteine by a color development-accelerating method.

Best Mode for Carrying out the Invention

In the method for quantitatively determining hydrogen sulfide or sulfide ions of the present invention, the case of utilizing the action of hydrogen sulfide or sulfide ions which inhibits the color development reaction between the metal ions and the metal indicator, will be described hereinbelow. That is, the hydrogen sulfide or sulfide ions present in a sample are allowed to contact with metal ions to form a metal sulfide, and at the same time, the metal ions are reacted with the metal indicator, and then the degree of color development is measured, whereby the amount of the complexes formed by the reaction between the metal ions and the metal indicator. Then, the above amount of the formed complexes is subtracted from the amount of the formed complexes when the metal ions are reacted

with the metal indicator under the same conditions as in the above provided that no hydrogen sulfide or sulfide ions present, whereby the decreased amount of the formed complexes is obtained by utilizing the characteristic that the metal sulfide forms no complexes. The decreased amount of the formed complexes corresponds to the amount of the hydrogen sulfide or sulfide ions present in the sample. Accordingly, the amount of the hydrogen sulfide or sulfide ions in the sample can be calculated based on the decreased amount of the formed complexes.

The case of utilizing the action of hydrogen sulfide or sulfide ions which accelerates the color development reaction between the metal ions and the metal indicator, will be described hereinbelow. That is, into the sample containing the hydrogen sulfide or sulfide ions, the metal ions and the metal indicator are added, and reaction is carried out, and then the degree of color development is measured to determine the amount of the formed complexes by the reaction between the metal ions and the metal indicator. Then, from this amount of the formed complexes, the amount of the formed complexes when the metal ions are reacted with the metal indicator under the same conditions as in the above provided that no hydrogen sulfide or sulfide ions present, is subtracted, whereby the increased amount of the formed complexes is determined. Since the increased amount of the formed complexes is attributable

to the acceleration of the reaction between the metal ions and the metal indicator by the hydrogen sulfide or sulfide ions, it corresponds to the amount of the hydrogen sulfide or sulfide ions. Accordingly, the amount of the hydrogen sulfide or sulfide ions in the sample can be calculated based on the increased amount of the formed complexes.

In the present invention, the metal ions are not particularly limited so long as the color development between the metal ions and the metal indicator is inhibited or accelerated by the hydrogen sulfide or sulfide ions. As the one inhibiting the color development reaction of the metal ions and the metal indicator, zinc ions are preferably used. As the one accelerating the above color development reaction, iron (II) and (III) ions are preferably used. Specifically, hydrochlorides, sulfates, acetates or the like of the above metals are used. However, there is no particular limitation so long as it dissolves in an aqueous solution and forms liberated metal ions.

As the metal indicator used in the present invention, there is no particular limitation so long as it is a substance of which the color development reaction with the metal ions is inhibited or accelerated by the hydrogen sulfide or sulfide ions. Preferred is the one having a high sensitivity of color development at the time of forming the complexes. For example, pyridylazo compounds and nitrosoaminophenol compounds are

preferably used. Further specifically, as the pyridylazo compounds, 2-(5-bromo-2-pyridylazo)-5-[N-N-propyl-N-(3-sulfopropyl)amino]phenol sodium salt (trade name: 5Br・PAPS; hereinafter simply referred to as 5Br・PAPS) and 2-(5-nitro-2-pyridylazo)-5-[N-N-propyl-N-(3-sulfopropyl)-amino]phenol sodium salt (trade name: Nitro・PAPS) are preferably used. Further, as the nitrosoaminophenol compounds, 2-nitroso-5-[N-N-propyl-N-(3-sulfopropyl)-amino]phenol (trade name: Nitroso・PSAP) and 2-nitroso-5-[N-ethyl-N-(3-sulfopropyl)amino]phenol (trade name: Nitroso・ESAP) are preferably used. These compounds are water soluble and have a property that they form complexes with zinc ions, copper ions, cobalt ions or iron ions and undergo color development with a high sensitivity. As these metal indicators, ones having various characteristics are commercially sold and available from, for example, Kabushiki Kaisha Dojin Kagaku Kenkyusho.

The specific substance in the present invention may be any substance so long as it forms hydrogen sulfide or sulfide ions by e.g. enzymatic reaction. For example, homocysteine and cysteine are preferably mentioned.

When the specific substance is homocysteine, as the component for forming hydrogen sulfide or sulfide ions from the specific substance, an enzyme (E1) which has a function of acting on homocysteine so that the homocysteine would form

hydrogen sulfide or sulfide ions, is used. As such an enzyme, for example, L-methionine-γ-lyase (enzyme No. EC class 4.4.1.11) and *O*-acetylhomoserine-lyase (enzyme No. EC class 4.2.99) may be mentioned. Particularly, *O*-acetylhomoserine-lyase may preferably be used.

L-methionine-γ-lyase is known as an enzyme that shows a decomposition (liberation) action against homocysteine and forms hydrogen sulfide in the absence of a thiol compound, but shows an action of catalyzing γ-substitution in the presence of a thiol compound. This enzyme is obtainable from microorganisms which are capable of producing it, for example, bacteria of Pseudomonas genus, but some types thereof may be commercially sold and available from, for example, Wako Junyaku Kabushiki Kaisha.

Further, *O*-acetylhomoserine-lyase is known as an enzyme that has an amino acid-synthesizing action (for example, an action of forming homocysteine from *O*-acetylhomoserine and hydrogen sulfide, and methionine from methanethiol) (reference can be made to "Handbook of Enzyme", compiled by Maruo et al, Asakura Shoten, 1982). The present inventors have found an enzymatic action that when *O*-acetylhomoserine-lyase is allowed to act on homocysteine in the presence of a thiol compound, hydrogen sulfide is formed by γ-substitution (Japanese Patent Application No. 10-347003).

With respect to *O*-acetylhomoserine-lyase, various

microorganisms that are capable of producing it have been known (reference can be made to, for example, Ozaki et al, J. Biochem 91; 1163-1171 (1982), Yamagata, J. Biochem 96; 1511-1523 (1984), Brzywczy et al, Acta. Biochimica. Polonica 40(3); 421-428 (1993)). *O*-Acetylhomoserine-lyase may be obtained by culturing these microorganisms, but some types thereof may be commercially sold and available from, for example, Yunitica Ltd. These enzymes (E1) have a function of strongly acting on homocysteine and acting a little on cysteine, thereby forming hydrogen sulfide.

Physicochemical properties of *O*-acetylhomoserine-lyase (trade name:"GCS") derived from Bacillus genus, manufactured by Yunitica Kabushiki Kaisha, are as follows. Among the following physicochemical properties, items other than molecular weight were determined by the present inventors.

Physicochemical Properties

1) Action: it catalyzes γ-substitution of L-homocysteine and a thiol compound, thereby forming hydrogen sulfide and a thiol compound-substituted homocysteine. Further, it catalyzes a substitution of L-methionine and a thiol compound, thereby forming methane thiol and a thiol compound-substituted homocysteine.

2) Substrate specificity: it acts on L-homocysteine and L-methionine. Further, it acts a little on L-cysteine by β-substitution.

3) Molecular weight: 180,000 (gel filtration method)

4) Optimum pH: 8.5-9.0

5) Km: 0.9 mM (L-homocysteine)

Further, when the specific substance is cysteine, as the component for forming hydrogen sulfide or sulfide ions from the specific substance, an enzyme (E2) which has a function of acting on cysteine so that the cysteine forms hydrogen sulfide or sulfide ions, is used. As such an enzyme, for example, *O*-acetylserine-lyase, β-cyanoalanine synthase and cysteine lyase may be mentioned. Particularly, *O*-acetylserine-lyase may preferably be used.

O-Acetylserine-lyase is known as an enzyme that has a cysteine-synthesizing action (an action of forming cysteine from *O*-acetylserine and hydrogen sulfide). The present inventors have found an enzymatic action that when *O*-acetylserine-lyase is allowed to act on cysteine in the presence of a thiol compound, hydrogen sulfide is formed by β-substitution (Japanese Patent Application No. 11-84035). This action is specific to cysteine. *O*-Acetylserine-lyase may be obtained from microorganisms that are capable of producing it (for example, Burnell et al, Biochim. Biophys. Acta 481; 246-265 (1977), Nagasaw et al, Methods Enzymol 143; 474-478 (1987), plants (for example, Droux et al, Arch.. Biochem. Biophys. 295(2); 379-390(1992), Yamaguchi et al, Biochim. Biophys. Acta 1251; 91-98(1995)), etc.

For example, the following are physicochemical properties of *O*-acetylserine-lyase obtained from spinach by the method described in Yamaguchi et al, Biochem. Biophys. Acta 1251; 91-98(1995). Among the following physicochemical properties, items other than molecular weight were determined by the present inventors.

Physicochemical Properties of *O*-acetylserine-lyase

1) Action: it catalyzes β-substitution of L-cysteine and a thiol compound, thereby forming hydrogen sulfide and a thiol compound-substituted cysteine.

2) Substrate specificity: it specifically acts on L-cysteine.

3) Molecular weight: 63,000 (gel filtration method)

4) Optimum pH: 9.0-11.0

5) Km: 0.27 mM (L-cysteine)

On the other hand, it has been known that when β-cyanoalanine synthase is allowed to act on cysteine in the presence of cyan, it shows a catalytic reaction of forming hydrogen sulfide by β-substitution, and when cysteine lyase is allowed to act on cysteine in the presence of sulfite ions, it shows a catalytic reaction of forming hydrogen sulfide by β-substitution.

As the thiol compound used in the present invention, there is no particular limitation so long as it can be used for a substitution reaction, and methane thiol, 2-

mercaptoethanol, dithiothreitol, thioglycerol and cysteamine may, for example, be mentioned. As the preferred ones, 2-mercaptoethanol and cysteamine may be mentioned.

The reaction in the method for measuring the hydrogen sulfide or sulfide ions of the present invention, is represented by the following Chemical Formulae 1 and 2.

Chemical Formula 1

Reaction A:

Metal ions + Metal indicator → Formation of complexes

(zinc) (pyridylazo compound) (color development)

Reaction B:

Metal ions + Sulfide ions

(zinc) (hydrogen sulfide)

→ Metal sulfide + Metal indicator —— (inhibition)

(zinc sulfide) (pyridylazo compound)

→ Formation of complexes (x)

Chemical Formula 2

Reaction C:

Metal ions + Metal indicator

(iron) (pyridylazo compound or nitrosoaminophenol compound)

—— (no reaction) → Formation of complexes (x)

Reaction D:

Metal ions + Sulfide ions + Metal indicator
(iron) (hydrogen sulfide) (pyridylazo compound or nitrosoaminophenol compound)
— (acceleration of reaction) → Formation of complexes (color development)

In the above Chemical Formula 1, Reaction A shows that in the absence of sulfide ions, the metal ions (for example, zinc ions) and the metal indicator (for example, a pyridylazo compound) form complexes rapidly and undergo color development.

Reaction B shows that by preliminarily allowing the metal ions (for example, zinc ions) to contact with sulfide ions to form a metal sulfide (for example, zinc sulfide), the formed compound (zinc sulfide) can not form complexes with the metal indicator, and the numerical value of color development decreases correspondingly. Accordingly, by calculating the decreased amount of the numerical value of color development, hydrogen sulfide or sulfide ions can be measured.

Namely, in the above Chemical Formula 1, it is important to select the combination of metal ions which form a stable metal sulfide by reacting with sulfide ions, and a metal indicator which forms complexes rapidly with the metal ions. As such a combination of the metal ions and the metal indicator, for example, the combination of zinc ions and a pyridylazo

compound may be mentioned. In the explanation hereinbelow, the measurement method utilizing the principle of the above Chemical Formula 1 is called a color development-inhibiting method.

Further, in the above Chemical Formula 2, Reaction C shows that, for example, by preliminarily allowing metal ions (e.g. iron ions) and a metal indicator (e.g. a pyridylazo compound or a nitrosoaminophenol compound) to co-present in a suitable buffer solution of from neutral to week alkaline condition (pH 7.0 - 9.0), the formation of complexes between the metal ions and metal indicator is inhibited and therefore no color development occurs.

Reaction D shows that in such a condition, when sulfide ions are added thereto, the formation of complexes thereof is accelerated corresponding to the concentration of sulfide ions, whereby the numerical value of color development is correspondingly increased. Accordingly, by calculating the increased amount of the numerical value of color development, the hydrogen sulfide or sulfide ions can be measured.

Namely, in the above Chemical Formula 2, it is important to control the conditions so that the metal ions and metal indicator hardly form complexes. As the combination of the metal ions and metal indicator, a combination of, for example, iron ions and a pyridyl compound or a nitrosoaminophenol compound, may be mentioned. In the explanation hereinbelow,

the measurement method utilizing the principle of the above Chemical Formula 2 is called a color development-accelerating method.

The reaction mechanism of the above color development-accelerating method may be considered as follows with respect to the combination of the iron ions and pyridylazo compound.

It is known that iron ions are present in various forms such as an aqua complex or a hydroxso complex in an aqueous solution and substantially affected by factors such as pH conditions. Further, under a high alkaline condition, formation of precipitates as hydroxides may occur. The following may be considered. In the combination of iron ions and a pyridylazo compound in this measurement system, by preliminarily allowing iron (II) and iron (III) ions to co-present in a suitable buffer solution of from neutral to a week alkaline condition (pH 7.0 - 9.0), the iron ions themselves form complexes in the solution, thereby inhibiting the reaction with the metal indicator (pyridylazo compound). Particularly, in the case of iron (II) ions, under the above condition, iron (II) ions are susceptible to oxidation by oxygen in addition to the formation of complexes, whereby the iron ions are present in the form of iron (III) ions. If sulfide ions are added to the iron ions in such condition and allowed to co-present together, iron (III) ions are reduced to iron

(II) ions by the reducing power of the sulfide ions, and become to be reactive with the pyridylazo compound, thereby undergoing color development. Further, some type of metal indicator hardly forms complexes with iron (III) ions, and in such a case, this principle of the acceleration method may be applied even if the pH is outside of the above-mentioned pH range.

Namely, according to the color development-accelerating method of the present invention, firstly, metal ions which become reactive with a metal indicator by the reduction with sulfide ions, are brought into a condition such that they are not reactive with the metal indicator by, for example, adjusting the conditions of the solution of metal ions appropriately. Then, by adding sulfide ions thereto, the metal ions in the non-reactive condition is changed to be reactive, and allowed to react with the metal indicator.

Accordingly, the color development-accelerating method of the present invention is accomplished by combining the above-mentioned metal ions with a suitable metal indicator.

Further, the reaction in the measurement method when the specific substance of the present invention is homocysteine and cysteine, is represented by the following Chemical Formula 3:

<u>Chemical formula 3</u>

Reaction E:

Homocysteine - (enzyme (E1)) → Hydrogen sulfide

Reaction F:

Cysteine - (enzyme (E2)) → Hydrogen sulfide

In the above Chemical Formula 3, Reaction E shows that hydrogen sulfide is formed by the action by an enzyme (E1) (for example, *O*-acetylhomoserine-lyase) which has a catalytic function of acting on homocysteine so that the homocysteine forms hydrogen sulfide. Further, Reaction F shows that hydrogen sulfide is formed by the action by an enzyme (E2) (for example, *O*-acetylserine-lyase) which has a catalytic function of acting on cysteine so that the cysteine forms hydrogen sulfide. Then, the formed hydrogen sulfide is measured by utilizing the reaction as shown in Chemical Formula 1 or 2 to quantitatively determine the homocysteine or cysteine.

Hereinbelow, the present invention will be described in further detail with reference to examples. However, it should be mentioned that the present invention is by no means restricted thereto.

Example 1 (Quantitative determination of sulfide ions by color development-inhibiting method)

As the sample and reagents, the following were used.

Sample: an aqueous solution containing from 0 to 100 μM of sodium sulfide (manufactured by Wako Junyaku K.K.) as sulfide ions.

1st reagent:

Tris buffer (pH 8.5) 100 mM

Zinc chloride 10 μM

2nd reagent:

5Br・PAPS (manufactured by Dojin Kagaku Kenkyusho K.K.) 1 mM

To 50 μl of the sample, 900 μl of the 1st reagent was added, and this was left to stand at room temperature for 5 minutes, and then 50 μl of the 2nd reagent was added thereto, and this was left to stand at room temperature for 5 minutes. Then, the absorbance of the reaction solution was measured with a wavelength of 550 nm. The results are shown in Fig. 1.

From the results of Fig. 1, decrease in the absorbance was confirmed corresponding to the concentration of the sulfide ions, and it was found that this correlation was quantitative. From the results, it was also found that sulfide ions can be measured by utilizing the inhibition reaction against the formation of complexes between the metal ions and metal indicator.

Example 2 (Quantitative determination of sulfide ions by color development-accelerating method)

As the sample and reagents, the following were used.

Sample: an aqueous solution containing from 0 to 100 μM of sodium sulfide (manufactured by Wako Junyaku K.K.) as sulfide ions.

1st reagent:

Tris buffer (pH 8.0) 100 mM

Ferrous chloride 33.3 μM

2-Mercaptoethanol 4 mM

2nd reagent:

5Br・PAPS (manufactured by Dojin Kagaku Kenkyusho K.K.) 0.25 mM

3rd reagent:

EDTA (pH 7.0) 200 mM

To 20 μl of the sample, 600 μl of the 1st reagent was added, and this was left to stand at 37 °C for 10 minutes, and then 160 μl of the 2nd reagent was added thereto, and this was left to stand at 37 °C for 5 minutes. Further, 20 μl of the 3rd reagent was added thereto, and this was left to stand at room temperature for 5 minutes, and then the absorbance of the reaction solution was measured with a wavelength of 550 nm. The results are shown in Fig. 2.

From the results of Fig. 2, increase in the absorbance was confirmed corresponding to the concentration of the sulfide ions, and it was found that this correlation was quantitative. From the results, it was also found that sulfide ions can be measured by utilizing the acceleration reaction for the formation of complexes between the metal ions and metal indicator.

In the following examples, as an enzyme (E1) to be used for the quantitative determination of homocysteine, *O*-acetylhomoserine-lyase derived from Bacillus genus (trade

name:"GCS", manufactured by Yunitica Ltd., the strength indicated below is a nominal value of the manufacturer) was used, and as an enzyme (E2) to be used for the quantitative determination of cysteine, *O*-acetylserine-lyase (derived from spinach) was used.

The *O*-acetylserine-lyase was prepared in accordance with the method of Yamaguchi et al (Biochim. Biophys. Acta 1251; 91-98 (1995)).

Specifically, through the extraction from 2 kg of spinach leaves and steps of ion exchange chromatography, hydrophobic chromatography and gel filtration chromatography, the enzyme of about 4,000 units was prepared and used for the examples. The strength was measured by the method described in the above document.

Example 3 (Quantitative determination of homocysteine by color development-accelerating method)

As the sample and reagents, the following were used.

Sample: an aqueous solution containing from 0 to 50 μM of L-homocystine (manufactured by Sigma K.K.) (as the L-homocysteine, from 0 to 100 μM).

1st reagent:

Tris buffer (pH 8.0) 100 mM

Ferrous chloride 33.3 μM

2-Mercaptoethanol 4 mM

O-Acetylhomoserine-lyase (manufactured by Yunitica Ltd. 3u/ml

2nd reagent:

5Br・PAPS (manufactured by Dojin Kagaku Kenkyusho K.K.) 0.25 mM

3rd reagent:

EDTA (pH 7.0) 200 mM

To 20 μl of the sample, 600 μl of the 1st reagent was added, and this was left to stand at 37 °C for 10 minutes, and then 160 μl of the 2nd reagent was added thereto, and this was left to stand at 37 °C for 5 minutes. Further, 20 μl of the 3rd reagent was added thereto, and this was left to stand at room temperature for 5 minutes, and then the absorbance of the reaction solution was measured with a wavelength of 550 nm. The results are shown in Fig. 3.

From the results of Fig. 3, increase in the absorbance was confirmed corresponding to the concentration of the homocysteine, and it was found that this correlation was quantitative. From the results, it was also found that homocysteine can be measured by utilizing the acceleration reaction for the formation of complexes between the metal ions and metal indicator.

Example 4 (Quantitative determination of cysteine by color development-accelerating method)

As the sample and reagents, the following were used.

Sample: an aqueous solution containing from 0 to 500 μM of L-cysteine (manufactured by Sigma K.K.).

1st reagent:

Tris buffer (pH 8.0) 100 mM

Ferrous chloride 33.3 μM

2-Mercaptoethanol 4 mM

O-Acetylserine-lyase (derived from spinach) 6 u/ml

2nd reagent:

5Br・PAPS (manufactured by Dojin Kagaku Kenkyusho K.K.) 0.25 mM

3rd reagent:

EDTA (pH 7.0) 200 mM

To 20 μl of the sample, 600 μl of the 1st reagent was added, and this was left to stand at 37 °C for 10 minutes, and then 160 μl of the 2nd reagent was added thereto, and this was left to stand at 37 °C for 5 minutes. Further, 20 μl of the 3rd reagent was added thereto, and this was left to stand at room temperature for 5 minutes, and then the absorbance of the reaction solution was measured with a wavelength of 550 nm. The results are shown in Fig. 4.

From the results of Fig. 4, increase in the absorbance was confirmed corresponding to the concentration of the cysteine, and it was found that this correlation was quantitative. From the results, it was also found that cysteine can be measured by utilizing the acceleration reaction

for the formation of complexes between the metal ions and metal indicator.

Industrial Applicability

As described above, according to the present invention, it is possible to quantitatively determine hydrogen sulfide or sulfide ions in a sample conveniently with high sensitivity. Further, it is also possible to quantitatively determine a specific substance in a sample conveniently with high sensitivity, by adding to a sample containing a specific substance, a component which acts on the specific substance so that the specific substance forms hydrogen sulfide or sulfide ions, and measuring the hydrogen sulfide or sulfide ions by the above quantitative determination method. Accordingly, the present invention can be utilized for, for example, a quantitative determination method of homocysteine, cysteine, etc. in a sample of a living body.

Claim(s):

1. A method for quantitatively determining hydrogen sulfide or sulfide ions, which comprises adding to a sample containing hydrogen sulfide or sulfide ions, metal ions or a compound which liberates said metal ions and a metal indicator which reacts with the metal ions and resultingly undergoes color development, wherein the color development is accelerated or inhibited by the hydrogen sulfide or sulfide ions; and measuring the degree of color development of the metal indicator.

2. The method according to Claim 1, wherein the metal ions are zinc ions or iron ions.

3. The method according to Claim 1, wherein the metal indicator is a pyridylazo compound or a nitrosoaminophenol compound.

4. A method for quantitatively determining a specific substance, which comprises adding to a sample containing a specific substance, a component which acts on the specific substance so that the specific substance forms hydrogen sulfide or sulfide ions, metal ions or a compound which liberates said metal ions, and a metal indicator which reacts with the metal ions and resultingly undergoes color development, wherein the color development is accelerated or inhibited by the hydrogen sulfide or sulfide ions; and measuring the degree of color development of the metal indicator.

5. The method according to Claim 4, wherein the metal ions are zinc ions or iron ions.

6. The method according to Claim 4, wherein the metal indicator is a pyridylazo compound or a nitrosoaminophenol compound.

7. The method according to Claim 4, wherein the specific substance is homocysteine, and the component which acts on the specific substance so that the specific substance forms hydrogen sulfide or sulfide ions, is an enzyme (E1) which acts on the homocysteine so that the homocysteine forms hydrogen sulfide

8. The method according to Claim 7, wherein the enzyme (E1) is a substance which catalyzes a substitution reaction to the homocysteine in the presence of a thiol compound.

9. The method according to Claim 8, wherein the enzyme (E1) is *O*-acetylhomoserine-lyase.

10. The method according to Claim 8, wherein the thiol compound is at least one selected from the group consisting of methane thiol, 2-mercaptoethanol, dithiothreitol, thioglycerol and cysteamine.

11. The method according to Claim 4, wherein the specific substance is cysteine, and the component which acts on the specific substance so that the specific substance forms hydrogen sulfide or sulfide ions, is an enzyme (E2) which acts on the cysteine so that the cysteine forms hydrogen sulfide.

12. The method according to Claim 11, wherein the enzyme (E2) is a substance which catalyzes a substitution reaction to the cysteine in the presence of a thiol compound.

13. The method according to Claim 12, wherein the enzyme (E2) is *O*-acetylserine-lyase.

14. The method according to Claim 12, wherein the thiol compound is at least one selected from the group consisting of methane thiol, 2-mercaptoethanol, dithiothreitol, thioglycerol and cysteamine.

Abstract

The present invention provides a method for quantitatively determining hydrogen sulfide or sulfide ions conveniently with high sensitivity, which comprises adding to a sample containing hydrogen sulfide or sulfide ions, metal ions or a compound which liberates said metal ions and a metal indicator which reacts with the metal ions and resultingly undergoes color development, wherein the color development is accelerated or inhibited by the hydrogen sulfide or sulfide ions; and measuring the degree of color development of the metal indicator. The present invention further provides a method for quantitatively determining a specific substance, which comprises adding to a sample containing a specific substance, a component which acts on the specific substance so that the specific substance forms hydrogen sulfide or sulfide ions, metal ions or a compound which liberates said metal ions, and a metal indicator which reacts with the metal ions and resultingly undergoes color development, wherein the color development is accelerated or inhibited by the hydrogen sulfide or sulfide ions; and measuring the degree of color development of the metal indicator.

Fig.1

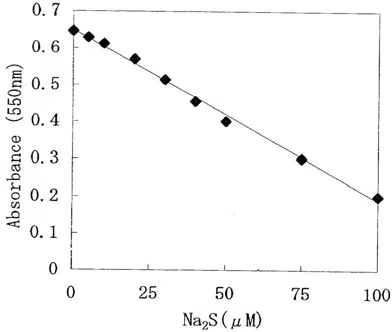

0. 7
0. 6
0. 5
0. 4
0. 3
0. 2
0. 1
0
Absorbance (550nm)
0
25
50
75
100
Na2S (μM)

Fig.2

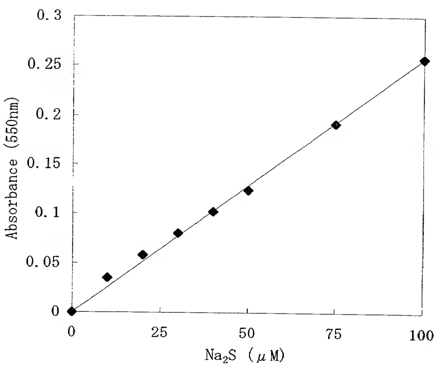

0. 3
0. 25
0. 2
0. 15
0. 1
0. 05
0
Absorbance (550nm)
0
25
50
75
100
Na2S (μM)

Fig.3

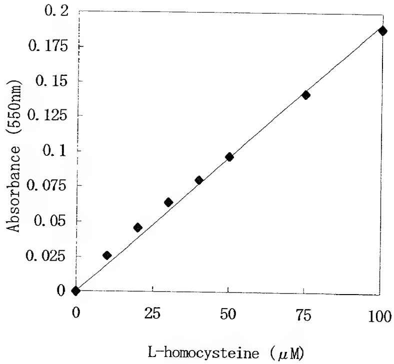
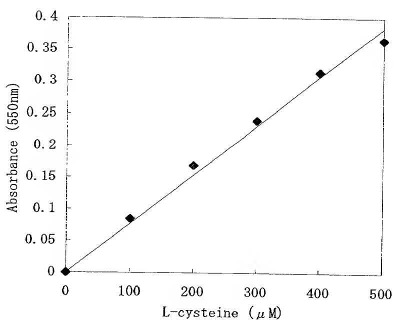

0. 2
0. 175
0. 15
0. 125
0. 1
0. 075
0. 05
0. 025
0
Absorbance (550nm)
0
25
50
75
100
L-homocysteine (μM)

Fig.4


0. 4
0. 35
0. 3
0. 25
0. 2
0. 15
0. 1
0. 05
0
Absorbance (550nm)
0
100
200
300
400
500
L-cysteine (μM)

10/96FHGL&C

U.S. Attorney Docket ___________

APPLICATION FOR UNITED STATES LETTERS PATENT

PCT Declaration and Power of Attorney (35 U.S.C. 371(c)(4))
PCT Application - United States Designated Office

As a below named inventor, I declare that:

My residence, post office address and citizenship are as stated below next to my name; I believe that I am the origi first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are lis below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:
Method for quantitatively determining hydrogen sulfide or sulfide ions, and method for quantitatively determining a specific substance utilizing it
described and claimed in International Application number PCT/JP99/06847 filed 07 December 1999
and, if it was amended, as amended on

I have reviewed and understand the contents of said specification, including claims.
I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR §1.56.

I claim priority benefits under 35 USC §119 of: (i) any foreign application(s) for patent or inventor's certificate listed below; or (ii) any United States provisional application(s) listed below; and have also identified below any foreign application(s) for patent or inventor's certificate, or PCT international application having a filing date before that of the application(s) on which priority is claimed.

COUNTRY	APPLICATION NUMBER	DATE (day, month, year)	PRIORITY CLAIMED
Japan	No.347003/1998	07,12,1998	yes X no
Japan	No.84035/1999	26,03,1999	yes X no
Japan	No.147848/1999	27,05,1999	yes X no

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

I appoint the following attorneys to prosecute this application and to transact all business in the U.S. Patent & Trademark Office connected therewith: Stephen H. Frishauf, Reg. No. 16,233; Leonard Holtz, Reg. No. 22,974; Herbert Goodman, Reg. No. 17,081; Thomas Langer, Reg. No. 27,264; Marshall J. Chick, Reg. No. 26,853; Walter J. Baum, Reg. No. 20,641; Richard S. Barth, Reg. No. 28,180; Douglas Holtz, Reg. No. 33,902; and Robert P. Michal, Reg. No. 35,614.

CORRESPONDENCE AND CALLS TO:

FRISHAUF, HOLTZ, GOODMAN, LANGER & CHICK, P.C.
767 Third Avenue - 25th Floor Tel.: (212) 319-4900
New York, New York 10017-2023 Fax.: (212) 319-5101

INVENTOR: SIGNATURE	DATE	RESIDENCE AND POST OFFICE ADDRESS
1-00 Sign: Hiroyuki Ebinuma	Date: 21 May 2001	Residence: (City & Country) Ryugasaki-shi,Ibaraki Japan JPX
Type: Hiroyuki Ebinuma	Citizen of: Japan	Post Office Address: c/o TSUKUBA R&D LABORATORIES, DAIICHI PURE CHEMICALS CO.,LTD., 3-1,Koyodai 3-chome,Ryugasaki-shi, Ibaraki,Japan
2-00 Sign: Koji Ushizawa	Date: 21 May 2001	Residence: (City & Country) Ryugasaki-shi,Ibaraki,Japan JPX
Type: Koji Ushizawa	Citizen of: Japan	Post Office Address: c/o TSUKUBA R&D LABORATORIES, DAIICHI PURE CHEMICALS CO.,LTD., 3-1,Koyodai 3-chome,Ryugasaki-shi, Ibaraki,Japan
Sign:	Date:	Residence: (City & Country)
Type:	Citizen of:	Post Office Address: